A taxonomic tool for identifying needle remains of south-western European *Pinus* species of the Late Quaternary

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This work provides a tool whereby the needle remains of native, south-western European *Pinus* spp. can be easily identified from species-specific epidermal features. To construct this tool, the needles of *P. uncinata*, *P. sylvestris*, *P. nigra*, *P. pinaster*, *P. pinea* and *P. halepensis* were gathered across the Northern Hemisphere range of each taxon and compared with non-indigenous trees growing in two South Australian Botanic Gardens. Three needles from each of these species were taken from three adult trees growing at three different localities. Light microscopy was used to observe the key epidermal and stomatal features of the needles. To improve interpretation, additional scanning electron microscopy samples were prepared. Epidermal features, including variation in the diameter of the epistomatal chamber aperture (pore), are described. A taxonomic key based on the size, shape and arrangement of the subsidiary cells of the stomatal complexes was constructed. This key enables the identification of pine needle fragments at the species level (except those belonging to the group *P.* gr. *nigra-uncinata*). Despite their overlapping range, pore size was helpful in distinguishing between *P. nigra* and *P. uncinata* and between three groups of species. Isolated stomata were also observed. Cluster and discriminant analyses of stomatal variables described in earlier studies were performed. Overlap in guard cell variables hampers species-level identification of isolated stomata. Species discrimination is improved if groups of ecological affinity are considered.

INTRODUCTION

In south-western Europe, species of *Pinus* L. are essential and emblematic trees for ecology and management because of their ability to occupy open spaces created by disturbance events (Richardson, 1998; Keeley, 2012). Although the study of natural archives provides information regarding the past responses to fire, climatic and anthropogenic disturbances for *Pinus* spp., these studies have been hampered by the small number of well-preserved pine cones that have been found, the only means by which these species can be reliably identified (Faegri & Iversen, 1989; Schweingrüber, 1990; Farjon & Styles, 1997). Pine needles and their fragments are also found at palaeobotanical sites (Stähli *et al.*, 2006; Finsinger & Tinner, 2007; Hilgartner, Nejako & Casey, 2009), and isolated stomata may also appear in palynological preparations (Aubert *et al.*, 2004; Froyd, 2005). The ability to identify such remains at the species level would be helpful to those trying to determine the local presence of different taxa (Ammann & Wick, 1993; Birks & Birks, 2000; MacDonald, 2001; Hicks, 2006) and those involved

in climate reconstruction studies (Lin, Jach & Ceulemans, 2001; Davis *et al.*, 2003; García-Amorena *et al.*, 2006; Rubiales *et al.*, 2010).

The epidermal morphology of *Pinus* needles has long been the subject of research (see Florin, 1931; Farjon, 1984; Boddi, Bonzi & Calamassi, 2002; Zellnig *et al.*, 2002). Such work has contributed to different phylogenetic proposals (Price, Liston & Strauss, 1998), but epidermal taxonomic information on its own allows only a few species to be reliably identified (see Kim, Whang & Hill, 1999; Ickert-Bond, 2000; Whang *et al.*, 2001; Whang, Kim & Hill, 2004; Boratyńska & Boratyński, 2007). Stružková (2002) and García Álvarez *et al.* (2009a, b) were among the first to provide an identification tool for European *Pinus* spp. based on needle epidermal features.

Six taxa of the genus *Pinus* are native to south-western Europe: *P. uncinata* Ramon ex DC., *P. sylvestris* L., *P. nigra* J.F.Arnold subsp. *salzmanii* (Dunal) Franco, *P. pinaster* Aiton, *P. pinea* L. and *P. halepensis* Mill. (Gaussen, Heywood & Chater, 1964; Castroviejo *et al.*, 1986). They occupy montane enclaves in Eurosiberian regions and a range of Mediterranean environments. All these taxa belong to *Pinus* subgenus *Pinus* and have two needles per dwarf-shoot that are striped with rows of stomata on the adaxial and abaxial surfaces (Mirov, 1967; Farjon, 1984; Ruiz de la Torre, 2006). Taxonomic studies based on needle epidermis features suggest that some of these species can be identified by Florin ring characteristics, the thickness of the epidermal cells or the shape and arrangement of the subsidiary cells of the stomatal complex (Yoshie & Sakai, 1985; Boratyńska & Bobowicz, 2001; Stružková, 2002; Boratyńska & Boratyński, 2007; García Álvarez *et al.*, 2009a, b). However, these investigations analysed different taxa separately or in small groups (Stružková, 2002), a single population per species (Yoshie & Sakai, 1985; García Álvarez *et al.*, 2009a, b) or cross-section features that could be poorly preserved in fossil material (Boratyńska & Bobowicz, 2001).

The aims of the present work were (1) to provide an identification key for the south-western European *Pinus* spp. that could be used to identify Late Quaternary needle remains; and (2) to improve the taxonomic identification of isolated *Pinus* stomata. This was accomplished by examining needle epidermal and stomatal features of different populations of *Pinus* spp. from across their worldwide distributions.

MATERIAL AND METHODS

Three natural populations of *P. uncinata*, *P. sylvestris*, *P. nigra*, *P. pinaster*, *P. pinea* and *P. halepensis* were analysed from across their ranges of distribution (Table 1; Fig. 1) (Gaussen *et al.*, 1964; Critchfield & Little, 1966; Castroviejo *et al.*, 1986; Martín Albertos, Díaz-Fernández & De Miguel y Del Ángel, 1998; Garbari, 2004). The sampled stands were selected to cover the maximum geographical distance and/or reported morphological and genetic variation. The populations of *P. pinea* included the two regions with the greatest genetic diversity (Lebanon and inland Iberia) reported by Vendramin *et al.* (2008). The stands of *P. pinaster* were located in the three regions (inland Iberian Peninsula, Atlas Mountains and Italian Peninsula) associated with the three mitochondrial haplotypes reported by Burban & Petit (2003) and belonged to three of the eight 'gene zones' reported by Bucci *et al.* (2007). The populations of *P. nigra* covered three of the five subspecies considered by Jalas & Suominen (1973): *P. nigra* subsp. *salzmannii*, *Pinus nigra* subsp. *nigra* J.F.Arnold and *Pinus nigra* subsp. *dalmatica* (Vis.) Businský.

Three needles were randomly sampled from three randomly selected adult trees per population. A 5-mm-long section of each needle was boiled in water for 1 h, macerated in Schulze's solution (Kerp, 1990), hand-cleaned with a lancet and mounted in glycerine on a microscope slide for observation by transmission light microscopy. Epidermal and stomatal analyses were performed using a digital camera and employing Leica Application Suite software (LAS version 3.2.0, Leica Microsystems).

The outer cuticle of some additional samples was examined by scanning electron microscopy (SEM) to clarify some epidermal features. The specimens were collected from natural European populations and from the Adelaide Botanic Park (South Australia) and the Mount Lofty Botanic Garden (Adelaide, South Australia) (Table 1). Samples were mounted on aluminium stubs with double-sided adhesive tape and air-dried. The stubs were then sputter coated with pure gold to a maximum thickness of 15 nm and examined using a Philips XL 20 scanning electron microscope operating at 10 kV, at Adelaide Microscopy (University of Adelaide).

Epidermal analysis involved the morphological description of the epidermal cells and the stomatal complex (the subsidiary cells and the pore or aperture of the epistomatal chamber) (Fig. 2). Photographic images of the epidermis were taken with a digital camera at 20 × 3.3 and 50 × 3.3 magnifications. The outlines were drawn using Adobe Photoshop Elements 6.0. Pore diameters were measured at 50 × 3.3 magnification directly from frozen video images using Leika Application Suite 3.2.0 software. The longest diameter of the pore (p) was recorded for ten pores per needle. To analyse the variance, all p values were log-transformed to meet the requirements of independence, normality and constant variance of the residuals (Sokal & Rohlf, 1995). Two analyses of vari-

Table 1. Populations sampled

Vegetal material for light microscopy observations

Id.	Taxon	Population	Leg. & Det.	Geographical coordinates
Pu1*	*Pinus uncinata*	Iberian Peninsula: Larra, Central Pyrenees (Spain)	Helios Sainz Ollero	42°56′N 0°48′W
Pu2	*Pinus uncinata*	Western Alps: Ibergeregg, (Switzerland)	Werner Suter	47°01′N 8°45′E
Pu3	*Pinus uncinata*	Iberian Peninsula: Barranco de Cregüeña, Central Pyrenees (Spain)	César Morales del Molino	42°39′N 0°36′E
Ps1*	*Pinus sylvestris*	Iberian Peninsula: Navacerrada, Guadarrama Range (Spain)	Javier Maldonado & authors	40°47′N 4°0′W
Ps2	*Pinus sylvestris*	Northern Europe: Koli National Park (Finland)	Rubén Manso González	63°06′N 29°49′E
Ps3	*Pinus sylvestris*	Siberia: Lake Baikal (Russia)	Mercedes García Antón & Miguel Ángel Casado	51°44′N 103°53′E
Pn1†	*Pinus nigra* subsp. *salzmannii*	Iberian Peninsula: La Sagra, Betic Cordillera (Spain)	Fernando G. Manzaneque & authors	37°57′N 2°34′W
Pn2	*Pinus nigra* subsp. *nigra*	Balkan Peninsula: Zmajevacki Potok (Serbia)	Srdjan Bojovic	43°52′N 19°25′E
Pn3	*Pinus nigra* subsp. *dalmatica*	Balkan Peninsula: Brac Island (Croatia)	Nera Marković & Dragica Žaja	43°18′N 16°37′E
Pt1†	*Pinus pinaster*	Iberian Peninsula: Ataquines, Northern Meseta (Spain)	Authors	41°13′N 4°43′W
Pt2	*Pinus pinaster*	Italic Peninsula: Monte Pisano, NW Toscana (Italy)	Rosaria Cartisano Unai Lopez de Heredia	43°46′N 10°32′E
Pt3	*Pinus pinaster*	Atlas Mountains: Tazaot (Morocco)	Juan Ruiz de la Torre; Javier Maldonado, Fernando G. Manzaneque, Felipe Martínez García & authors	35°14′N 5°06′W
Pp1†	*Pinus pinea*	Iberian Peninsula: Biar, Levante (Spain)	Authors	38°30′N 0°44′W
Pp2	*Pinus pinea*	Iberian Peninsula: Ataquines, Northern Meseta (Spain)	Authors	41°13′N 4°43′W
Pp3	*Pinus pinea*	Ramlieh, El Chouf Mountains (Lebanon)	Pedro Regato Pajares	33°45′N 35°39′E
Ph1†	*Pinus halepensis*	Iberian Peninsula: Maigmó, Levante interior (Spain)	Authors	38°30′N 0°37′W
Ph2	*Pinus halepensis*	Atlas Mountains: Chaambi National Park, Kasserine (Tunisia)	Pedro Regato Pajares	35°11′N 8°39′E
Ph3	*Pinus halepensis*	Balkan Peninsula: Brac Island (Croatia)	Nera Marković & Dragica Žaja	43°21′N 16°42′E

Vegetal material used in the SEM observations

Taxon	Natural population	Leg. & Det.	Geographical coordinates
Pinus uncinata	Iberian Peninsula: Baqueira, Central Pyrenees (Spain)	Authors	42°42′N 0°57′E
Pinus sylvestris	Northern Europe: Koli National Park (Finland)	Rubén Manso González	63°06′N 29°49′E
Pinus nigra subsp. *salzmannii*	Iberian Peninsula: Alto Tajo, Iberian Range (Spain)	G. Manzaneque, César Morales del Molino & authors	40°42′N 2°11′W

Table 1. *Continued*

Vegetal material used in the SEM observations

Taxon	Natural population	Leg. & Det.	Geographical coordinates
Pinus pinaster	Iberian Peninsula: Luzaga, Iberian Range (Spain)	César Morales del Molino	40°58′N 2°25′W
Pinus pinea	Iberian Peninsula: Ataquines, Northern Meseta (Spain)	Authors	41°13′N 4°43′W
Pinus halepensis	Iberian Peninsula: Barranco del Baúl, Baetic Mountains (Spain)	Authors	37°24′N 2°51′W

Taxon	Origin	Geographic Coordinates
Pinus sylvestris	Mount Lofty Botanic Garden, Adelaide Hills (Australia)	34°59′S 138°43′E
Pinus nigra	Mount Lofty Botanic Garden, Adelaide Hills (Australia)	34°59′S 138°43′E
Pinus pinaster	Mount Lofty Botanic Garden, Adelaide Hills (Australia)	34°59′S 138°43′E
Pinus pinea	Adelaide Botanic Garden, Adelaide (Australia)	34°55′S 138°36′E
Pinus halepensis	Adelaide Botanic Garden, Adelaide (Australia)	34°55′S 138°36′E

*Material previously studied by García Álvarez *et al.* (2009b).
†Material previously studied by García Álvarez *et al.* (2009a).

ance involving the nested factors *species*, *population*, *tree* and *needle* were then undertaken: (1) a variance components analysis of random effects (Sokal & Rohlf, 1995; McDonald, 2009), performed using Microsoft Office Excel, 2007 (Supporting Information file 'spreadsheets_nestedanova.xls'), which provided information on the contribution of each factor to the variability as a whole; and (2) a linear mixed effects model (LME) analysis employing the factor *species* as a fixed effect factor and the remaining factors as random effects factors (Bates & Maechler, 2009; R Development CoreTeam, 2009), which was used to make pairwise comparisons between the means of the groups defined by the factor *species* (Crawley, 2007). For predictive analysis, identification ranges for the maximum pore diameter (p) were established for the homogeneous groups defined in the latter analysis (LME), using the 95th percentiles of the estimated normal distributions for each taxon (mean ± 2SD).

Stomatal analysis was based on the measurement of the thickenings of the guard cells (see glossary of morphological terms in the Appendix). Sixteen variables (Fig. 3) reported in earlier studies (Trautmann, 1953; Hansen, 1995; Sweeney, 2004; García Álvarez *et al.*, 2009a, b) were measured for ten stomata per needle, following the same procedure described for variable p.

Two kinds of multivariate analysis were then performed. (1) For understanding the general behaviour of the measured variables, the spontaneous grouping of stomata, needles and trees was studied by cluster analysis of single measures and by the cluster analysis of means of stomatal variables for needles and trees [UPGMA method with Euclidean distances; PC-ORD v.4.0 software (McCune & Mefford, 1999)]. (2) For establishing their taxonomic value, discriminant analysis (SPSS v.15.0.1 software) was performed to observe the grouping of stomata by species. This last analysis determined the taxonomic contribution of each variable, providing a primitive predictive tool for the classification of unknown stomata.

To provide a more useful classification tool with less uncertainty, such that palaeobotanical sediments might be ecologically (Ozenda & Borel, 2000) and chronologically characterized, new discriminant analyses were performed with different subsets of data. These subsets were defined in terms of the ecological affinity of species (Gaussen *et al.*, 1964; Farjon, 1984; Castroviejo *et al.*, 1986; Costa Tenorio, Morla Juaristi & Sainz Ollero, 1997; Ruiz de la Torre, 2006). Subset 1 involved the group of montane pines (i.e. the Supramediterranean/Subalpine unit), including *P. uncinata*, *P. sylvestris* and *P. nigra*; subset 2 involved the thermophilous pines (i.e. the

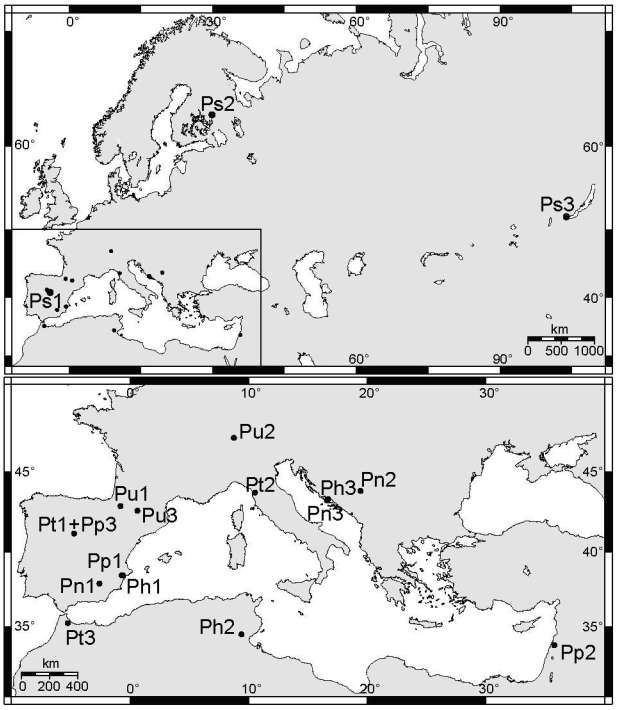


Figure 1. Location of the sampled populations (see Table 1). Pu1, Pu2 and Pu3, *Pinus uncinata*; Ps1, Ps2 and Ps3, *Pinus sylvestris*; Pn1, Pn2 and Pn3, *Pinus nigra*; Pt1, Pt2 and Pt3, *Pinus pinaster*; Pp1, Pp2 and Pp3, *Pinus pinea*; Ph1, Ph2 and Ph3, *Pinus halepensis*.

Thermomediterranean unit), including *P. halepensis*, *P. pinea* and *P. pinaster*; and subset 3 involved the mesomediterranean pines (i.e. the Mesomediterranean unit), including *P. nigra*, *P. pinaster*, *P. pinea* and *P. halepensis*.

RESULTS

Epidermal features

Taxonomic differences in the stomatal complexes of each species were observed (Fig. 2, Table 2). The size, shape and arrangement of the subsidiary cells identified five taxonomic groups: *P. sylvestris*, *P. pinaster*, *P. pinea*, *P. halepensis* and *P.* gr. *nigra-uncinata*.

Pinus sylvestris could be identified by its ring structure. The presence of isomorphic or slightly elongated polar subsidiary cells characterizes *P. pinaster*. *Pinus halepensis* was distinguished by having a large number of lateral subsidiary cells (three or four per side). Irregularity in size and shape of the subsidiary cells identified *P. pinea*. In this species the subsidiary cell contours and pores were usually unclear under the light microscope and the SEM samples revealed strong thickenings and furrows (Fig. 2Ap). Together, all these features provided the support for the dichotomous key shown in the Appendix.

Despite the treatments to which the needle fragments were subjected, parts of the epidermis of almost all the needles of *P. pinaster*, *P. halepensis*, *P. nigra* and *P. uncinata* retained a thick waxy coating and, in these areas, the epistomatal chambers were full of wax. These wax plugs were less abundant in *P. pinea* and *P. sylvestris*, especially in needles collected from trees in northern stands of *P. sylvestris* (Ps2 and Ps3).

Pore size

Pinus pinaster and *P. halepensis* had the largest pore sizes, followed by *P. nigra*. *Pinus uncinata* had small pores and *P. pinea* and *P. sylvestris* had very small pores (Fig. 4). *Species* was shown to be the principal factor (68.3%) determining pore size variance. The other factors, in decreasing order, are *residuals* (due to intra-needle variability, 15.8%), *population* (8.4%), *tree* (3.9%) and *needle* (3.6%) (see Table 3).

The t-values of pairwise comparisons of the LME model suggested three homogeneous groups: (a) *P. sylvestris* and *P. pinea*; (b) *P. pinea* and *P. uncinata*; and (c) *P. nigra*, *P. pinaster* and *P. halepensis* (Table 4). The predictive analysis performed for these groups using the 95th percentile of the ln(p)-estimated normal distributions of each species returned three identification ranges [a: $p < 16.43$ μm] [a,b,c: 16.43 μm $< p <$ 38.94 μm] and [c: $p < 38.94$ μm] (Fig. 4). Given the difficulty in distinguishing *P. uncinata* and *P. nigra* from one another based on their qualitative epidermal features (Table 2), their discrimination intervals were also calculated (*P. uncinata*: $p < 21.94$ μm; *p. nigra*: $p > 38.94$ μm).

Stomatal features

The values of the measured stomatal variables reflected the expected high variability (Supporting Information Table S1). Cluster analysis of the stomatal data showed a weak trend towards species segregations (Supporting Information Fig. S1). The cluster analyses of the means of stomatal variables for trees and needles (Fig. 5, Supporting Information Fig. S2) provided higher grouping trends for species and populations, but this information was still insufficiently strong to allow reliable species discrimination. *Pinus pinea* was the species that showed the best segregation from the others. *Pinus nigra* and *P. pinaster* showed the weakest taxonomic segregations.

Joint discriminant analysis (Supporting Information Fig. S3) revealed clearer species separation trends than did the cluster analyses (Fig. 5, Supporting Information Figs S1 and S2). Five discriminant functions with significant Wilk's λ values explained 100% of the variance using 15 of the 16 stomatal variables (Supporting Information Table S2). Simple

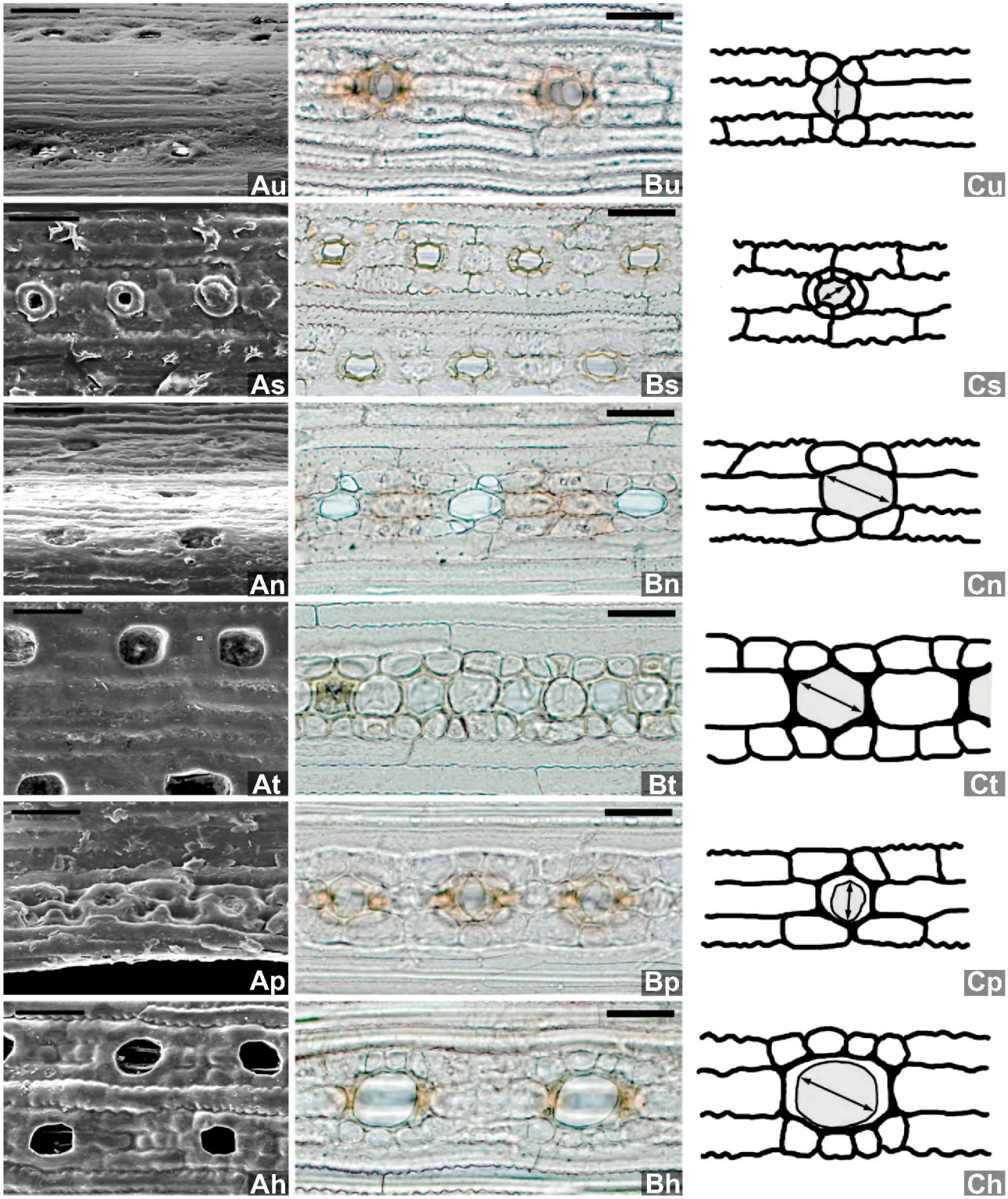


Figure 2. Stomatal complexes. A, outer cuticle observed by SEM. Scale bar = 50 µm. B, epidermis preparations observed by light microscopy. C, simplified diagram of the main features observed by light microscopy. u (Au, Bu, Cu), *Pinus uncinata*; s (As, Bs, Cs), *P. sylvestris*; n (An, Bn, Cn), *P. nigra*; t (At, Bt, Ct), *P. pinaster*; p (Ap, Bp, Cp), *P. pinea*; h (Ah, Bh, Ch), *P. halepensis*. Arrow: variable *p*. Scale bar = 50 µm.

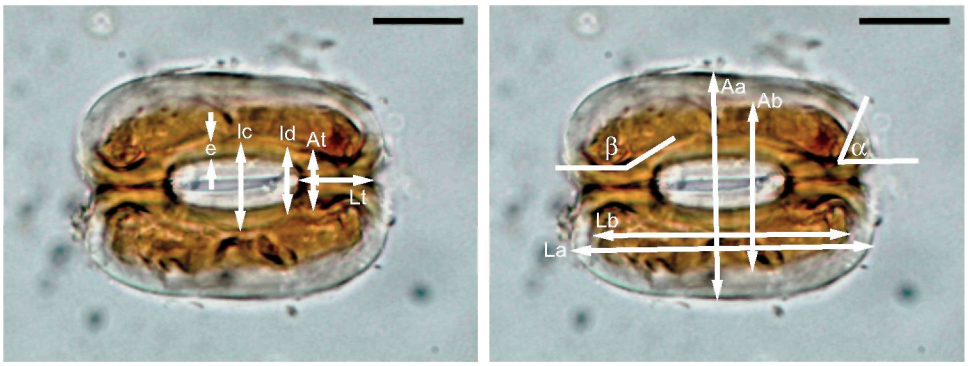


Figure 3. Stomatal variables: stomatal width (*Aa*); stomatal length (*La*); upper woody lamella width (*Ab*); upper woody lamella length (*Lb*); medial lamella border width (*e*); stem length (*Lt*); stem width (*At*); angle of attachment of upper woody lamella (*alpha*, α); angle between the stem and the closest medial lamella border (*beta*, β); distance between the external limits of the medial lamellae borders measured at the centre (*lc*); distance between the external limits of the medial lamellae borders measured at the point where both meet to form the stem (*ld*); stomatal width ratio (*coef_a* = *Aa*/*La*); upper woody lamellar width ratio (*coef_b* = *Ab*/*Lb*); coefficient associated with the shape of the medial lamellae borders (*coef_c* = *lc*/*ld*); coefficient associated with the relative width of the medial lamellae borders of a guard cell with respect to the distance between the external limits of the medial lamellae borders (*coef_e* = *lc*/*e*); stem width ratio (*coef_T* = *At*/*Lt*).

and cross validation of Fisher's classification functions returned 60.7 and 59.6% correct classification rates, respectively.

Discriminant analysis involving the montane pines data (*P. uncinata*, *P. sylvestris* and *P. nigra*) produced distinct clouds of points (Fig. 6A). Two discriminant functions with eight stomatal variables were identified. Fisher's classification functions (Table 5) generated 73.8 and 73.1% correct classifications in simple and cross validations, respectively.

The discriminant analysis cloud points for the thermophilous pines (*P. halepensis*, *P. pinea* and *P. pinaster*) also suggested the three taxa involved could be distinguished (Fig. 6B). The two discriminant functions made use of seven variables. Fisher's classification functions (Table 6) returned 84.1 and 83.3% correct classifications in simple and cross-validations, respectively.

The discriminant analysis of the mesomediterranean pines (*P. nigra*, *P. pinaster*, *P. pinea* and *P. halepensis*) showed a slightly less well-defined segregation (Fig. 6C). Three discriminant functions involving eight variables were detected. Fisher's classification functions (Table 7) returned 72.5 and 71.9% correct classification rates in simple and cross-validations, respectively.

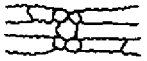
DISCUSSION

Epidermal features

This work provides a means of identifying pine needle fragments at the species level for the six *Pinus* spp. in south-western Europe and confirms the results of previous studies that dealt with more limited material (García Álvarez *et al.*, 2009a, b). Qualitative epidermal features (shape, relative size and arrangement of the cells of the stomatal rows) allow *P. halepensis*, *P. pinea*, *P. pinaster*, *P. sylvestris* and the group *P.* gr. *nigra-uncinata* to be clearly distinguished (see the dichotomous key in the Appendix). The cuticular coating of plants is generally highly resistant to degradation (Kerp, 1990), and thus the epidermal 'footprints' of fossilized leaves provide a means by which fossil plants can be identified (Barclay *et al.*, 2007).

Pinus nigra and *P. uncinata* can often be distinguished, as their needle epidermis (and cuticles) show subtle differences in pore size and the number of lateral subsidiary cells (also recorded in the key in the Appendix). If an unknown needle is well preserved, identification can be confirmed by cross-sectional features such as the thickness of the epidermis and the location of resin canals (Sutherland, 1934; Boratyńska & Bobowicz, 2001; Boratyńska & Boratyński, 2007). The similarity of the stomatal complexes of *P. nigra* and *P. uncinata* justifies the inclusion of these species in a single category. This is coherent with old and new phylogenetic classifications showing them to belong to the same section or subsection (Shaw, 1914, 1924; Pilger, 1926; Critchfield & Little, 1966; Klaus, 1989; Price *et al.*, 1998; Gernandt *et al.*, 2005; Kaundun & Lebreton, 2010). These older classifications also include *P. sylvestris* and *P. mugo* Turra in the same group, section or subsection as *P. nigra* and *P. uncinata*. *Pinus mugo* is not only phylogenetically close to *P. uncinata* (Gernandt *et al.*, 2005), but it is also morphologically similar (Gaussen *et al.*, 1964). The stomatal complex of *P. mugo* has a structure similar to that

Table 2. Epidermal features

	Pinus uncinata	*Pinus sylvestris*	*Pinus nigra*	*Pinus pinaster*	*Pinus pinea*	*Pinus halepensis*
						
Stomatal row	Slightly higher width between stomata.	Constant width.	Slightly greater width between stomata.	Approximately constant width.	Approximately constant width.	Slightly narrower width between stomata.
	Strong size and shape differences between elements (cells, pores).	Strong size and shape differences between elements (cells, pores).	Strong size and shape differences between elements (cells, pores).	Slight size and shape differences between elements (cells, pores).	Strong size and shape differences between elements (cells, pores).	Strong size and shape differences between elements (cells, pores).
Pore	14–38 µm Elliptical–polygonal.	10–26 µm Circular.	20–45 µm Elliptical–polygonal.	24–55 µm Polygonal–circular.	12–30 µm Irregularly elliptical.	24–52 µm Elliptical.
Outline of the epistomatal chamber	Polygonal with rounded vertices, close to the pore.	Circular, the same as the pore.	Polygonal with rounded vertices, close to the pore.	Polygonal with sharp vertices, close to the pore, well-defined walls.	Polygonal with sharp vertices, larger than the pore, blurred walls.	Polygonal with rounded vertices, slightly larger than the pore.
Arrangement of subsidiary cells	Lateral and polar cells with no ring structure.	Clear ring structure.	Lateral and polar cells with no ring structure.	Lateral and polar cells with no ring structure.	Lateral and polar cells with no ring structure.	Lateral and polar cells with no ring structure.
Lateral subsidiary cells	2(3) per side. Isomorphic.	(1)2(3) per side. Isomorphic.	2–3 per side. Isomorphic.	2(3) per side. Isomorphic.	2(3) per side. Irregular: elliptical, ovate or isomorphic.	3–4 per side. Isomorphic.
	Smaller than the pore, much smaller than the other cells of the stomatal row.	Smaller than the pore, much smaller than the other cells of the stomatal row.	Smaller than the pore, much smaller than the other cells of the stomatal row.	Smaller than the pore, similar in size and shape to the other cells of the stomatal row.	Very irregular, similar to or larger than the pore, similar in size to the other cells of the stomatal row.	Smaller than the pore, much smaller than the other cells of the stomatal row.
Polar subsidiary cells	1 per side. Elongated lengthwise.	1 per side. Elongated crosswise, annular fragment shaped.	1 per side. Elongated lengthwise.	1 per side. Isomorphic or elliptical lengthwise.	1 per side. Lengthwise elongated, often wider at the centre and narrower at the poles.	1 per side. Elongated lengthwise.
	Larger than the lateral subsidiary cells, similar to the other cells of the stomatal row.	Larger than the lateral subsidiary cells, smaller than the other cells of the stomatal row.	Larger than the lateral subsidiary cells, similar to the other cells of the stomatal row.	Larger than the lateral subsidiary cells and the other cells of the stomatal row.	Larger than the lateral subsidiary cells and the other cells of the stomatal row.	Larger than the lateral subsidiary cells, similar to the other cells of the stomatal row.

of *P. uncinata* and *P. nigra*, as revealed by the comparison of the present results with those of Stružková (2002). However, the stomatal complex of *P. sylvestris* is different in appearance.

Yoshie & Sakai (1985) classified the Florin ring of *Pinus* subgenus *Pinus* needles into four different types, and reported those of *P. sylvestris* to be Type D (very marked). Strikingly, among the species these authors analysed, this feature is only shared with *P. densiflora* Siebold & Zucc., the species phylogenetically closest to *P. sylvestris* (Eckert & Hall, 2006).

The peculiarities of the stomatal rows of *P. pinea* (Fig. 2, Table 2) are consistent with traditional phylogenetic classifications that segregate this taxon into a monospecific group (Little & Critchfield, 1969; Klaus, 1989; Price *et al.*, 1998). However, later classifications that incorporate genetic and biogeochemical information include *P. halepensis* and *P. pinaster* in the same group (Liston *et al.*, 1999; Wang *et al.*, 1999; Gernandt *et al.*, 2005; Kaundun & Lebreton, 2010). Finally, the most recent proposal (Grivet *et al.*, 2013), using genetic and life-history trait data, confirms the phylogenetic proximity of *P. pinea* to the rest of the pines in section *Pinus* subsection *Pinaster* (Gernandt *et al.*, 2005) and suggests the heterogeneity of this assembly in terms of adaptation strategies. *Pinus pinea* has been reported to show unusual and exclusive morphological and biogeographical features such as a large, edible kernel and an 'umbrella' crown shape (Vendramin *et al.*, 2008; Grivet *et al.*, 2013). Its characteristic stomatal complex can be added to the list of elements absent in other closely related pines (Farjon, 1984).

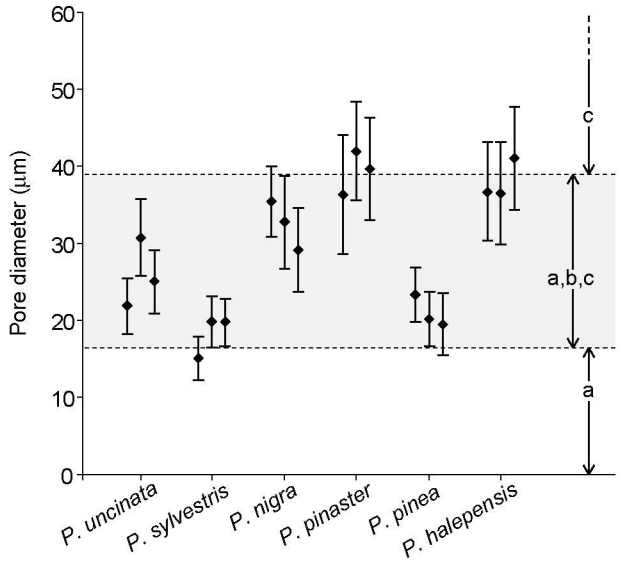


Figure 4. Maximum diameter of the pore (variable *p*, µm). Error bars: 2SD. Grey: overlapping area for the six species.

Some of the observed cuticle features are of interest for future work. Epicuticular wax was found to be extremely abundant in nearly all of the analysed samples. This allows gaseous exchange to occur while reducing water loss (Farjon & Styles, 1997). According to Yoshie & Sakai (1985), in *Pinus* pore size and wax production are directly related. Evidence of this relationship was detected in the present work, with wax-blocked pores more commonly observed in the four taxa with the highest *p* values (*P. uncinata*, *P. nigra*, *P. pinaster* and *P. halepensis*). Finally, the present observations indicate differences in stomatal density (SD) between the studied taxa, i.e. the larger number of stomata per row and stomatal rows per needle in *P. pinaster*. Although SD has usually been linked to environmental factors such as atmospheric CO_2 concentration (Lin *et al.*, 2001), the inclusion of SD in systematic studies of *Pinus* should be considered.

Stomatal features

Pinus stomata are often revealed in pollen preparations via the characteristic thickenings of their guard cells (Trautmann, 1953; Esau, 1982; Ammann & Wick, 1993; Hansen, 1995; Sweeney, 2004). The present results confirm the difficulty of identifications at the species level given the absence of distinctive, qualitative stomatal features.

Table 3. Results for nested ANOVA of ln(*p*) values

	Sum of squares	d.f.	Mean square	Fs	*P*	Variance component	Var. comp. (percentage)
Among species	138.3926	5	27.6785	20.9890	0.000015	0.0976289	68.33
Populations within species	15.8246	12	1.3187	5.4796	0.000033	0.0119784	8.38
Trees within populations	8.6637	36	0.2407	3.2754	1.11E-6	0.0055728	3.90
Needles within trees	7.9354	108	0.0735	3.2503	1.10E-23	0.0050869	3.56
Within needles (resid.)	32.9597	1458	0.0226			0.0226061	15.82
Total	203.7760	1619					100.00

Table 4. Pairwise comparisons of the species using the linear mixed effects model (Bates & Maechler, 2009) employing ln(p) values

		P. uncinata	*P. sylvestris*	*P. nigra*	*P. pinaster*	*P. pinea*	*P. halepensis*
P. uncinata	Estimate	**3.23**	−0.35	0.23	0.43	−0.21	0.39
	Std. error	**0.07**	0.10	0.10	0.10	0.10	0.10
	t value	**43.64**	−3.36	2.21	4.04	*−1.96*	3.75
P. sylvestris	Estimate	0.35	**2.88**	0.58	0.77	0.15	0.74
	Std. error	0.10	**0.07**	0.10	0.10	0.10	0.10
	t value	3.36	**38.89**	5.57	7.40	*1.40*	7.12
P. nigra	Estimate	−0.23	−0.58	**3.46**	0.19	−0.44	0.16
	Std. error	0.10	0.10	**0.07**	0.10	0.10	0.10
	t value	−2.21	−5.57	**46.77**	*1.83*	−4.17	*1.54*
P. pinaster	Estimate	−0.43	−0.77	−0.19	**3.65**	−0.63	−0.03
	Std. error	0.10	0.10	0.10	**0.07**	0.10	0.10
	t value	−4.04	−7.40	*−1.83*	**49.35**	−6.00	*−0.28*
P. pinea	Estimate	0.21	−0.15	0.44	0.63	**3.03**	0.60
	Std. error	0.10	0.10	0.10	0.10	**0.07**	0.10
	t value	*1.96*	*−1.40*	4.17	6.00	**40.87**	5.72
P. halepensis	Estimate	−0.39	−0.74	−0.16	0.03	−0.60	**3.62**
	Std. error	0.10	0.10	0.10	0.10	0.10	**0.07**
	t value	−3.75	−7.12	*−1.54*	*0.28*	−5.72	**48.95**

95% confidence critical t value: 1.968822. Values in italics: under the threshold of 95% confidence. Values in bold: intercept.

By contrast, the discriminant analysis performed with the six taxa together returned low correct species classification rates based on stomatal measurements (Fig. 3). Indeed, the high stomatal variability observed suggests that all isolated stomata should be measured, and other sources of palaeobotanical information explored (e.g. cuticles, charcoals) if more reliable identifications are to be made. When interpreting data, the possibilities of rapid migration rates or unexpected refugia (e.g. García-Amorena *et al.*, 2007; Morales-Molino *et al.*, 2011) should be considered.

However, with both previous considerations, a statistically improved species identification tool for groups of ecological affinity is provided. This tool is based on the evidence that, in certain areas, the species can be defined as being potentially present during a given time period.

For example, *Pinus* stomata can always be attributed to *P. sylvestris* in Scottish and Scandinavian Late Quaternary records as it is the only *Pinus* sp. that lived there in that period (Gervais *et al.*, 2002; Froyd, 2005). Similarly, the number of potential *Pinus* spp. can be reduced in some south-western European regions, increasing the confidence of stomatal-based classification (in the present work from 60 to 70–80%). In the Eurosiberian mountain regions of the Iberian Peninsula (Bolòs, 1985; Rivas-Martinez, 1983; Allué Andrade, 1990), the presence of *P. halepensis*, *P. pinea* and *P. pinaster* during the Quaternary would be unexpected (Rubiales *et al.*, 2010). Thus, the three taxa are generally ignored in the discussions of these sites (e.g. Aubert *et al.*, 2004; Pélachs *et al.*, 2011).

Similarly, in strongly thermophilous Mediterranean environments (i.e. the Thermomediterranean bioclimatic belt; Ozenda, 1975; Quézel & Barbero, 1985; Rivas-Martinez, 1983), the pines most likely to be present would be *P. halepensis*, *P. pinea* and *P. pinaster* (Costa Tenorio, Morla Juaristi & Sainz Ollero, 1997). Therefore, discussion regarding the presence of *Pinus* could be reduced to these three species in the most thermophilous assemblages described in Iberian southern sites (e.g. Carrión *et al.*, 2010). However, in cooler Iberian Mediterranean environments (i.e. the Mesomediterranean bioclimatic belt; Rivas-Martinez, 1983), *P. nigra* should be included in the discussions (e.g. Carrión *et al.*, 2010; García-Antón *et al.*, 2011).

Population relationships

Although the weak trend towards species and population segregation observed in the cluster analysis of the stomatal data (Supporting Information Fig. S1) could be related to stochastic processes, the cluster analyses using mean values for the stomatal variables (Fig. 5, Supporting Information Fig. S2) reveals relationships between stomatal morphology and the regional origin of certain populations. This could be indicating an ecotypic differentiation among populations and/or dif-

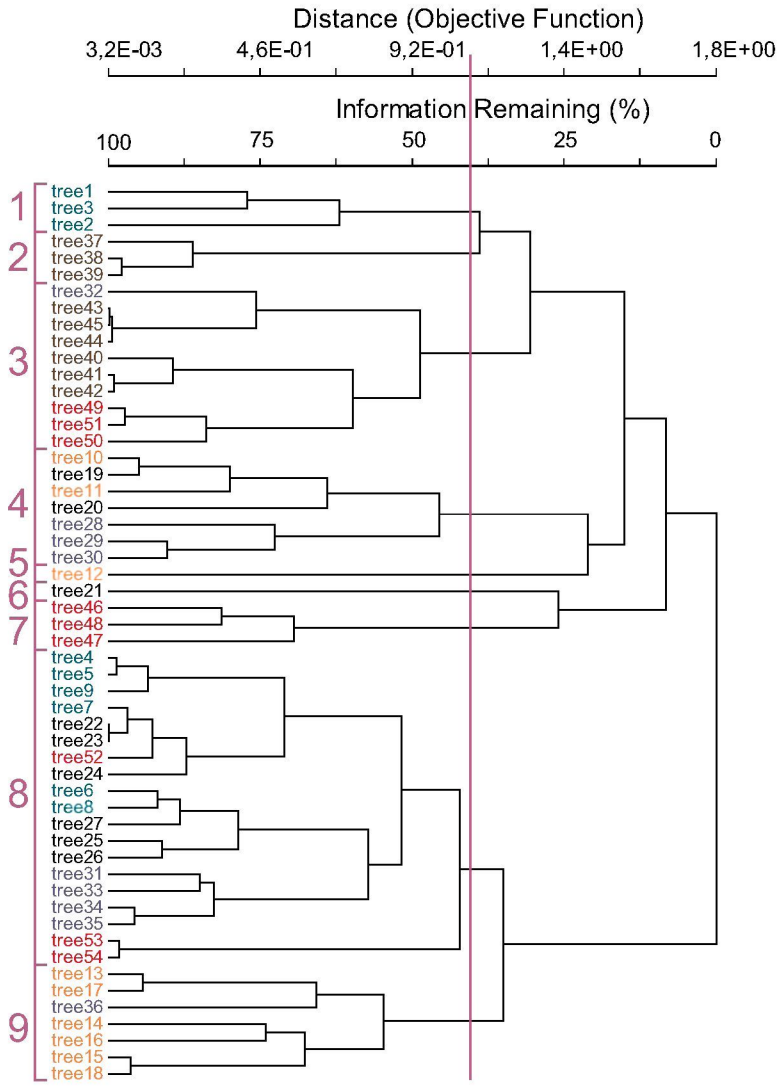


Figure 5. Cluster analysis dendrogram using tree mean values and UPGMA distances. *P. uncinata*: Pu1: 1–3; Pu2: 4–6; Pu3: 7–9. *P. sylvestris*: Ps1: 10–12; Ps2: 13–15; Ps3: 16–18. *P. nigra*: Pn1: 19–21; Pn2: 22–24; Pn3: 25–27. *P. pinaster*: Pt1: 28–30; Pt2: 31–33; Pt3: 34–36. *P. pinea*: Pp1: 37–39; Pp2: 40–42; Pp3: 43–45. *P. halepensis*: Ph1: 46–48; Ph2: 49–51; Ph3: 52–54.

ferent morphological population responses to different environmental conditions (phenotypic plasticity) (Chambel *et al.*, 2005; Soto *et al.*, 2010).

For example, the observed distance between the Iberian population (Ps1) and the northern populations (Ps2, Northern Europe; Ps3, Siberia; Fig. 5) of *P. sylvestris* can be understood as a combination of high phenotypic plasticity due to contrasting water balance conditions (Poyatos *et al.*, 2007) and to genetic variation, which is greater in the Iberian populations (Cheddadi *et al.*, 2006; Prus-Głowacki *et al.*, 2012). Genetic diversity and phenotypic plasticity have also been reported for *P. pinaster* (Gómez *et al.*, 2005; Chambel, Climent & Alía, 2007) and *P. nigra* (Gaussen *et al.*, 1964; Zara & Richard, 2007; Soto *et al.*, 2010). The population patterns shown in Figure 5 support these findings. The segregation of *P. halepensis* populations by means of stomatal values (Fig. 5) is most probably due to marked phenotypic plasticity (Chambel *et al.*, 2007) and ecotypic variability in different adaptive traits (Climent *et al.*, 2008; Voltas *et al.*, 2008; Santos-Del-Blanco *et al.*, 2013) rather than low genotypic diversity (Chambel *et al.*, 2007; Soto *et al.*, 2010).

Cluster analyses of the mean values for the needle- and tree-associated stomatal variables showed the

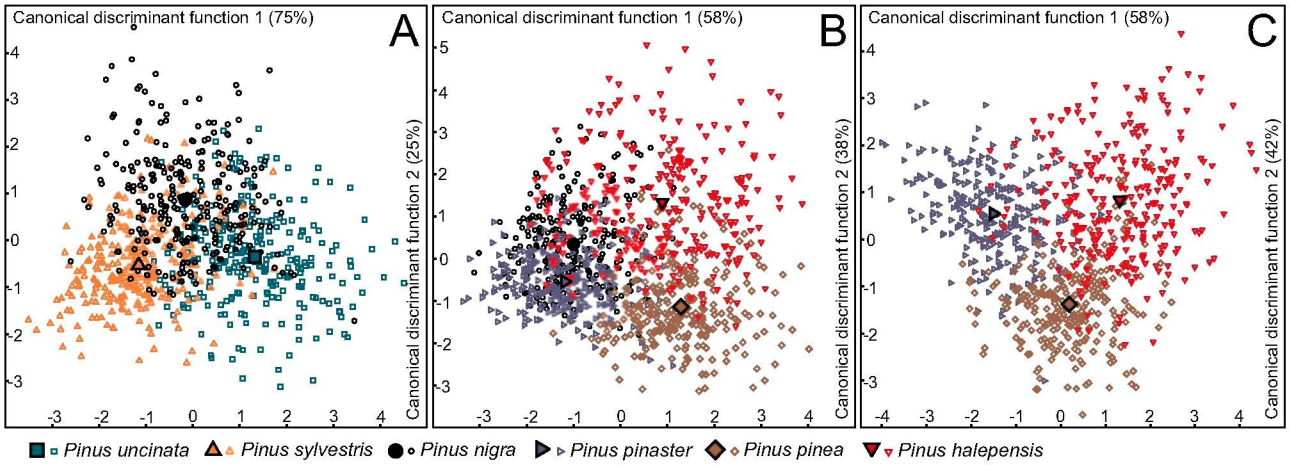


Figure 6. Discriminant analysis plots. A, montane pines. B, thermophilous pines. C, Mediterranean pines. Larger symbols: centroids.

Table 5. Fischer's classification function coefficients (montane pines)

	P. uncinata	*P. sylvestris*	*P. nigra*
Aa	1.263	0.795	0.871
La	1.091	1.076	1.297
Lb	6.903	6.648	6.820
coef_b	285.612	276.191	285.622
coef_c	128.128	123.465	130.511
At	–13.269	–13.070	–14.119
coef_t	194.393	201.315	211.520
beta	2.719	2.642	2.706
(Constant)	–628.536	–574.785	–619.808

Measured values for each variable are multiplied by the coefficients for each species to obtain an overall final value for each column. Unknown stomata belong to the species whose column of coefficients produces the highest final value.

Table 6. Fischer's classification function coefficients (thermophilous pines)

	P. pinaster	*P. pinea*	*P. halepensis*
Aa	1.456	1.727	1.502
La	3.098	3.019	3.477
Ab	0.099	0.506	0.222
coef_b	180.916	168.617	175.097
lc	–1.634	–1.858	–1.763
Lt	4.036	4.487	3.706
coef_t	92.696	80.645	86.551
(Constant)	–270.081	–281.223	–288.093

Measured values for each variable are multiplied by the coefficients for each species to obtain an overall final value for each column. Unknown stomata belong to the species whose column of coefficients produces the highest final value.

Pyrenean population Pu3 to be much more similar to the Alpine Pu2 population than to the Pyrenean Pu1 population of *P. uncinata* (Fig. 5, Supporting Information Fig. S2). The first probable explanation for this is that although the Pyrenean populations (Pu1 and Pu3) are geographically close, the environmental settings in which they grow are markedly different. The westernmost (Pu1) population grows in a possibly pseudo-marginal, karst environment (Costa Tenorio *et al.*, 1997). On the other side, the cluster analysis results for *P. uncinata* support the findings of Dzialuk *et al.* (2009) who, when unable to find a clear geographical pattern in plastid markers in this species, suggested that gene flow between the Alpine and Pyrenean populations had not been completely absent during the Quaternary.

Finally, the exceptional species segregation of *P. pinea* (Fig. 5, Supporting Information Figs S1 and S2) fits the scant genetic diversity reported by Vendramin *et al.* (2008) and the low phenotypic plasticity observed for different adaptive traits (Chambel *et al.*, 2007; Mutke *et al.*, 2010).

CONCLUSIONS

Light microscopy examination of the epidermal features of the needles of native *Pinus* spp. in southwestern Europe can be used to differentiate each species. The main differences lie in the shape and arrangement of the subsidiary cells of the stomatal complexes. A key is proposed that allows users to reliably classify needle fragments of *P. sylvestris*, *P. pinaster*, *P. pinea*, *P. halepensis* and *P.* gr. *nigra*-

Table 7. Fischer's classification function coefficients (mesomediterranean pines)

	P. nigra	*P. pinaster*	*P. pinea*	*P. halepensis*
Aa	2.039	2.160	2.490	2.261
La	0.678	0.550	0.488	0.923
Ab	1.106	0.941	1.093	0.940
Lb	1.078	0.997	1.148	1.074
ld	−6.212	−5.962	−6.248	−6.050
coef_e	3.855	4.577	4.094	4.315
Lt	7.893	8.049	8.462	7.680
coef_t	201.688	196.632	185.336	189.453
(Constant)	−234.860	−228.678	−248.318	−250.916

Measured values for each variable are multiplied by the coefficients for each species to obtain an overall final value for each column. Unknown stomata belong to the species whose column of coefficients produces the highest final value.

uncinata. *Pinus nigra* and *P. uncinata* can be differentiated by studying the frequency of single lateral subsidiary cells and the size of the pores.

The present stomatal analysis revealed the wide variability of variables related to the thickenings of the guard cell walls. Stomata-based species identification remains a challenge; isolated stomata on pollen slides can be identified with 70–80% confidence, but only when other sources of information allow the definition of reduced subsets of potential species.

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APPENDIX

Dichotomous key for identifying epidermis fragments of south-western European *Pinus* species

1 Subsidiary cells arranged in a ring surrounding the pore. Small pore size (*c.* 10–26 μm): ***P. sylvestris***

1 Subsidiary cells not arranged in a ring. Variable pore size: **2**

2 Three or four subsidiary cells on either side of the pore. Stomatal row somewhat wider at the position of each stoma. Large pore (*c.* 24–52 μm): ***P. halepensis***

2 Two subsidiary cells (sometimes one or three) on either side of the pore. Stomatal row of uniform width or slightly narrower at stomatal locations. Pore size variable: **3**

3 Length of the polar subsidiary cells not much greater than the length of the lateral subsidiary cells: **4**

3 Length of the polar subsidiary cells more than double that of the lateral subsidiary cells: **5**

4 Stomatal rows homogeneous in appearance (slight size and shape differences among subsidiary cells, other cells and pores). Outline of the cells reveals rounded vertices; epistomatal chamber with well-defined walls. Large pores (*c.* 24–55 μm): ***P. pinaster***

4 Stomatal rows relatively heterogeneous in appearance. Vertices of cells and pores more angular than rounded, with slightly blurred walls. Small pore (*c.* 12–30 μm): ***P. pinea***

5 Outline of the epistomatal chamber traces a larger area than the pore, with vertices more sharp than rounded. Polar subsidiary cells of variable length. Small pore (*c.* 12–30 μm): ***P. pinea***

5 Pore outline similar to that of the epistomatal chamber, which has rounded vertices. Polar subsidiary cells elongated, as are the other cells of the stomatal row. Pore size variable: ***P. gr nigra-uncinata***

- Commonly two lateral subsidiary cells on either side of the pore, sometimes three, rarely one. Medium–small pore size (*c.* 14–38 μm): ***P. uncinata***
- Commonly three subsidiary cells on either side of the pore, sometimes two, never just one. Medium–large pore size (*c.* 20–45 μm): ***P. nigra***

Glossary of morphological terms based on the terminology of Florin (1931), Trautmann (1953), Stace (1965), Hansen (1995), Macdonald (2001) and Sweeney (2004)

Florin ring: A circular thickening formed by the cells surrounding the stomata of pine needles, first

described by Florin (1931). Six different types of Florin ring have been described for the genus *Pinus*, four of which (types A–D) are seen in the subgenus *Pinus* (Yoshie & Sakai, 1985; Farjon & Styles, 1997).

Lamella (woody lamella): Lignified portions of the upper and lower wall of the guard cells. The upper lamella is often thicker than the lower. The lower woody lamella is not often preserved in fossil pollen samples. In the genus *Pinus*, the outline of the guard cells coincides with the shape of the lower woody lamella; the latter completely covers the lower wall of the cell (see Fig. 3).

Medial lamellae border: Portion of the lamellae bordering the stoma, often thickened; close to a line drawn through the stems (see Fig. 3).

Pore: The aperture of the epistomatal chamber (Fig. 2). In many conifers the guard cells are very deeply sunken and overarched by the subsidiary cells, so that in surface view their position is marked by a ring of subsidiary cells around a nearly circular hole.

Stem: The portion of the lamellae borders beginning at their junction and extending towards the poles away from the stoma (see Fig. 3).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Simplified cluster analysis dendrogram using stomata values and UPGMA distances. Pu, *P. uncinata*; Ps, *P. sylvestris*; Pn, *P. nigra*; Pt, *P. pinaster*; Pp, *P. pinea*; Ph, *P. halepensis*.
Figure S2. Cluster analysis dendrogram using needle mean values and UPGMA distances. Blue, *P. uncinata*; orange, *P. sylvestris*; dark green–black, *P. nigra*; violet, *P. pinaster*; brown, *P. pinea*; red, *P. halepensis*. Distribution of needles by population:

Pu1: 1–9	Ps1: 28–36	Pn1: 55–63	Pt1: 82–90	Pp1: 109–117	Ph1: 133–144
Pu2: 10–18	Ps2: 37–45	Pn2: 64–72	Pt2: 91–99	Pp2: 118–126	Ph2: 145–153
Pu3: 19–27	Ps3: 46–54	Pn3: 73–81	Pt3: 100–108	Pp3: 127–135	Ph3: 154–162

Figure S3. Plot of the stomata values by discriminant functions 1 and 2. The larger symbols represent the group centroids.
Table S1. Stomatal data: average and standard deviation of every studied population.
Table S2. Standardized canonical discriminant function coefficients.
Spreadsheets_nestedanova.xls. An Excel file with the spreadsheets used to perform the variance components analysis of random effects (Sokal & Rohlf, 1995; McDonald, 2009).